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(54) Title: PURIFIED SAPONINS AS ORAL ADJUVANTS

(57) Abstract

The use of purified saponins, e.g., QS-21, as oral adjuvants is described. Also described are compositions, e.g., immunogenic and vaccine compositions, particularly for oral administration, comprising purified saponins.

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PURIFIED SAPONINS AS ORAL ADJUVANTS

BACKGROUND OF THE INVENTION

The immune system uses many mechanisms for attacking pathogens; however, not all of these mechanisms are necessarily activated after immunization. Protective immunity induced by vaccination is dependent on the capacity of the vaccine to elicit the appropriate immune response to resist or eliminate the pathogen. Depending on the pathogen, this may require a cell-mediated and/or humoral immune response.

The current paradigm for the role of helper T cells in the immune response is that T cells can be separated into subsets on the basis of the cytokines they produce, and that the distinct cytokine profile observed in these cells determines their function. This T cell model includes two major subsets: TH-1 cells that produce IL-2 and interferon γ (IFN-γ) which augment both cellular and humoral immune responses, and TH-2 cells that produce IL-4, IL-5 and IL-10 which augment humoral immune responses (Mosmann *et al.*, *J. Immunol.* *126*:2348 (1986)).

It is often desirable to enhance the immunogenic potency of an antigen in order to obtain a stronger

immune response in the organism being immunized and to strengthen host resistance to the antigen-bearing agent. A substance that enhances the immunogenicity of an antigen with which it is administered is known as an adjuvant. For example, certain lymphokines have been shown to have adjuvant activity, thereby enhancing the immune response to an antigen (Nencioni *et al.*, *J. Immunol.* *139*:800-804 (1987); EP285441 to Howard *et al.*).

It has generally been observed that ingestion of inactivated or killed infectious agents only results in a secretory immune response, if indeed any immune response is raised at all; few compounds are known to potentiate the immune response to orally administered antigens (Maharaj *et al.*, *Can. J. Microbiol.* *32*:414-420 (1986)). Compounds which have been identified as capable of acting as an adjuvant for orally administered antigens have produced variable responses ranging from moderately effective to ineffective, depending on the antigen used and mode of delivery.

SUMMARY OF THE INVENTION

Work described herein assessed the ability of a purified saponin, e.g., QS-21, delivered orally or intranasally, to serve as an adjuvant to induce an immune response to an antigen, e.g., tetanus toxoid (TT) or ovalbumin (OVA). As described herein, a purified saponin, QS-21, has been used in oral immunizations with TT and OVA, and QS-21 has exhibited adjuvant activity by this delivery route.

The compositions of the present invention, comprising a purified saponin and an antigen, modulate the protective immune response to the antigen; that is, the vaccine composition is capable of quantitatively and/or qualitatively improving the vaccinated host's antibody response, and increasing cell-mediated immunity

for a protective response to a pathogen. This can be accomplished, for example, by increasing the numbers of antibodies produced upon immunization with the antigen (e.g., a quantitative improvement), or by altering the profile of the immune response, such as from a Th1 response to a Th2 response (e.g., a qualitative improvement).

The invention also pertains to methods for eliciting or increasing a host's humoral and/or cell-mediated immune response, comprising administering to a vertebrate host an effective amount of an immunogenic composition comprising an antigen and a purified saponin in suspension in a physiologically acceptable solution. In a preferred embodiment the purified saponin is QS-21.

The invention also pertains to methods for eliciting or increasing a vaccinate's humoral and/or cell-mediated immunity, for a protective immune response, comprising administering to a vertebrate host an effective amount of a vaccine composition comprising an antigen and a purified saponin in suspension in a physiologically acceptable solution. In a preferred embodiment the purified saponin is QS-21.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B illustrate the TT-specific IgG (Figure 1A) and IgM (Figure 1B) antibody titers at specific intervals after co-oral immunization with TT and varying doses of QS-21.

Figure 2 shows an analysis of the immunoglobulin subclasses produced after co-oral immunization with TT and varying doses of QS-21.

Figures 3A and 3B show the differential effect of QS-21 doses observed for IgE responses. Responses were detected for both polyclonal (Figure 3A) and TT-specific (Figure 3B) IgE responses with 50 μg QS-21 at day 7, and

lower or no IgE responses were detected with 100 and 500 μg of QS-21, respectively.

Figure 4 shows an analysis of fecal extracts indicating that 3 of 5 mice generated mucosal anti-TT IgA titers of 1:64 at the 50 μg dose of QS-21, and minimal IgA responses were detected at the 100 or 500 μg dosage.

Figure 5 shows the total serum IgG and subclass endpoint titers at day 42 after administration of 20 μg TT alone, or 20 μg TT given intranasally with 20 or 40 μg of QS-21 to 3 groups of 5 mice. Doses of 15 μl were given in PBS on day 0, 14, and 28.

Figure 6 shows the serum TT-specific IgA endpoint titers after administration of 20 μg TT alone, or 20 μg TT given intranasally with 20 or 40 μg of QS-21 to 3 groups of 5 mice. Doses of 15 μl were given in PBS on day 0, 14, and 28.

Figures 7A and 7B show the presence of TT-specific IgA (Figure 7A) and IgG (Figure 7B) in vaginal wash samples after administration of 20 μg TT alone, or 20 μg TT given intranasally with 20 or 40 μg of QS-21 to 3 groups of 5 mice. Doses of 15 μl were given in PBS on day 0, 14, and 28.

Figure 8 shows that both spleen and Peyer's patches secrete both Th1 and Th2 cytokines after C57BL/6 mice were orally immunized at days 0, 7, and 14 with 250 μg tetanus toxoid and 50 μg QS-21.

Figures 9A and 9B show a comparison of cholera toxin (CT) adjuvant and QS-21 adjuvant as oral adjuvants for raising serum antibody titers. Figure 9A shows IgM, IgA and IgG titers, which are generally comparable between the two adjuvants (although serum IgA is slightly lower with QS-21 immunization). Figure 9B shows serum IgG isotypes.

Figures 10A and 10B show the serum IgE response (Figure 10A) and reciprocal PCA titer (Figure 10B), measured at days 7, 14, and 21, induced by cholera toxin and QS-21.

Figure 11 shows an analysis of fecal extracts and vaginal washes for tetanus toxoid-specific IgA.

Figure 12 shows the mucosal and systemic immune responses to ovalbumin co-orally administered with QS-21.

DETAILED DESCRIPTION OF THE INVENTION

Saponins are glycosidic products composed of a ring structure (the aglycone) to which is attached one or more sugar chains. The saponins are grouped together based on several common properties. In particular, saponins are surfactants which display hemolytic activity and form complexes with cholesterol. Saponins are also somewhat structurally diverse, in that the aglycone can be asteroid, triterpenoid or a steroidalalkaloid, and the number of sugars attached to the glycosidic bonds vary. Saponins are found mainly in plants but have also been found in certain marine animals, particularly echinoderms such as starfish and sea cucumbers.

Work described herein relates to the utility of purified saponins as oral adjuvants. Accordingly, this invention pertains to compositions, e.g., vaccine compositions, comprising an antigen and a purified saponin. In a particular embodiment, the purified saponin is QS-21. In another embodiment the purified saponin is selected from the group consisting of QS-7, QS-17, QS-18, QS-21 and combinations thereof. These vaccine compositions modulate the protective immune response to the antigen; that is, the vaccine composition is capable of eliciting the vaccinated

host's cell-mediated immunity for a protective response to the pathogenic antigen.

Purified saponins can be produced from crude saponin extracts or partially purified saponin fractions obtained from a variety of sources. The term "extract" is intended to encompass both liquid and solid forms. For example, the saponin can be obtained from plants of the genus *Chrysanthellum* (see, for example, U.S. Patent 4,335,113), from the South American tree *Quillaja saponaria* (e.g., *Quillaja saponaria Molina*) (see, for example, U.S. Patent 5,057,540), from *Aesculus hippocastanum* seeds (see, for example, U.S. Patent No. 5,118,671), from *Glyccyrrhiza glabra* (see, for example, U.S. Patent 5,147,859), from *Centella asiatica* and *Terminalia sp.* (see, for example, U.S. Patent 5,166,139) and from *Chenopodium quinoa* (see, for example, U.S. Patent 5,597,807).

As used herein, "purified saponin" is intended to mean a substantially pure saponin which is purified to one or more of the following standards: 1) appearing as only one major carbohydrate staining band on silica gel TLC (EM Science HPTLC Si60) in a solvent system of 40 mM acetic acid in chloroform/methanol/water (60/45/10, v/v/v); 2) appearing as only one major carbohydrate staining band on reverse phase TLC (EM Science Silica Gel RP-8) in a solvent system of methanol/water (70/30, v/v); or 3) appearing as only one major peak upon reverse-phase HPLC on Vydac C4 (5 μm particle size, 330 Å pore, 4.8 mm ID X 25 cm L) in 40 mM acetic acid in methanol/water (58/42,v/v).

Saponin preparations can be purified from a suitable source or extract using methods known in the art, such as column chromatography, HPLC, immunoadsorbent techniques, affinity chromatography and immunoprecipitation (see, for example, U.S. Patent Nos.

5,057,540 and 4,501,734). For example, the preparation of substantially pure saponins from aqueous extracts of *Quillaja saponaria Molina* bark is described in U.S. Patent 5,057,540, the teachings of which are incorporated herein by reference in their entirety.

Purified saponin preparations, such as Stimulon® QS-21, QS-7, QS-17 and QS-18 (also known as QA-21, QA-7, QA-17 and QA-18, respectively) are also commercially available (Aquila Biopharmaceuticals, Inc., Worcester, MA). Also useful in the present invention are biologically active subunits or fragments of purified saponins. The invention also encompasses the use of natural and pharmaceutically acceptable salts of purified saponins of the invention.

The antigen of this invention, e.g., a bacterial antigen such as tetanus toxoid antigen, can be combined with one or more purified saponins according to the invention, and this composition can be used to elicit an immune response to the antigen in a vertebrate such as a mammalian host. For example, the antigen can be a tetanus toxin or ovalbumin antigen or a portion thereof which retains the ability to stimulate an immune response. The ability of portions of a given antigen to stimulate an immune response can be assessed by art-recognized methods, such as enzyme immunoassay against specific peptides within a portion to detect antibodies that recognize that particular portion (humoral response) or a delayed-type hypersensitivity assay against specific peptides in that portion to detect cell-mediated immune response thereto.

The term "antigen" is intended to include a molecule which contains one or more epitopes which stimulate a host's immune system to produce a humoral, cellular and/or secretory immunological response. The antigen of the invention can be a subunit antigen, as

well as killed, attenuated or inactivated bacteria, viruses, protozoa, fungi, parasites or other microbes. The antigen can be, for example, a protein, peptide, polysaccharide, lipid or DNA antigen.

Suitable antigens can be derived from, for example, Pasteurella, Actinobacillus, Haemophilus, Salmonella and Eimeria species, as well as rotaviruses, herpes viruses (e.g., BHV-1, EHV-1, PRV, parvovirus, rabiesvirus, influenza viruses, parainfluenza viruses, hepatitis viruses, HIV cornaviruses, tumor antigens, hormones, hormone analogs and the like.

The immunological adjuvant activity of a purified saponin, alone or with a particular antigen, can be assessed using methods known in the art, such as ELISAs, hemagglutination assays and neutralization assays. As used herein, "immunological adjuvant activity" is intended to mean the ability to potentiate an immunological response in a host to which the saponin and antigen are administered. Typically the purified saponin will be administered with the antigen, either in the same admixture or composition (see, for example, International Publication No. WO 93/05789), or at the same time but in a separate composition or formulation; however, the purified saponin can be administered prior to or subsequent to the administration of the antigen. Alternatively, the purified saponin can be orally administered in the absence of a particular antigen to elicit a non-specific immune response. Adjuvant activity includes, but is not limited to, the ability to enhance the immunological response to the antigen by increasing the immunogenicity of the antigen or by reducing the dose or level of antigen required to produce an immune response.

As used herein, an "immune response" or "immunological response" to a particular antigen (or a

non-specific immune response) is intended to include the production of a secretory, cellular, humoral or antibody-mediated response to the antigen (or a generalized response). The manifestation of the response in the immunized host can include the production of antibodies (e.g., IgA, IgD, IgE, IgG or IgM antibodies), proliferation of B and/or T lymphocytes, stimulation of cytotoxic T lymphocytes that recognize antigen-presenting cells, expansion of T cell populations and the potentiation of signals which cause differentiation, activation or growth of cells of the immune system.

Typically the administration of the purified saponin adjuvants of the invention will cause or result in an enhanced immune response to an antigen of interest. In this context, "enhanced" is intended to mean that the immune response to the antigen is greater in the presence of the purified saponin than in the absence of the purified saponin. Comparisons of immune responses in the presence and absence of purified saponin adjuvants can be performed by routine methods, such as antibody titer comparisons by radioimmunoassay or ELISA of saponin-adjuvanted compositions and appropriate controls.

Purified saponins can be used as described herein as adjuvants to enhance the immunological response to an antigen or to elicit a non-specific immune response. In a particular embodiment the purified saponin is an oral adjuvant. For example, the purified saponin can be used in a composition to immunize a mammal against a particular pathogen or subunit antigen, or to prime an immune response to a particular antigen.

The method of the present invention comprises administering to a mammal, particularly a human or other primate, an immunologically effective dose of a vaccine

composition comprising an antigen, e.g., a tetanus toxoid antigen, and an adjuvant amount of a purified saponin, e.g., QS-21. As used herein, an "adjuvant amount" or an "effective amount" of purified saponin is intended to mean an amount which enhances an immune response to a coadministered antigen, or an amount which stimulates non-specific immunity in the absence of antigen. For example, doses of from about 0.5 μg to about 500 μg, and more particularly from about 10 μg to about 100 μg will typically be effective to provide an adjuvant effect; however, variations in these dosage ranges will occur depending upon the particular purified saponin. Moreover, the particular dosage will depend upon the age, weight and medical condition of the mammal to be treated, as well as on the method of administration. Suitable doses will be readily determined by the skilled artisan.

The vaccine composition can be optionally administered in a pharmaceutically or physiologically acceptable vehicle, such as physiological or phosphate buffered saline, water, dextrose, ethanol polyols (such as glycerol or propylene glycol), and combinations thereof. A small amount of detergent may also be included to enhance vaccine stability.

The vaccine composition may optionally comprise additional components, such as buffering agents, preservatives, emulsifying agents and adjuvants, such as vegetable oils or emulsions thereof, surface active substances, e.g., hexadecylamin, octadecyl amino acid esters, octadecylamine, lysolecithin, dimethyl-dioctadecylammonium bromide, N,N-dicoctadecyl-N'-N'bis (2-hydroxyethyl-propane diamine), methoxyhexadecylglycerol, and pluronic polyols; polyamines, e.g., pyran, dextransulfate, poly IC, carbopol; peptides, e.g., muramyl dipeptide,

dimethylglycine, tuftsin; immune stimulating complexes; oil emulsions; lipopolysaccharides such as MPL® (3-O-deacylated monophosphoryl lipid A (RIBI ImmunoChem Research, Inc., Hamilton, Montana); mineral gels, and immunostimulating oligonucleotides. The antigens of this invention can also be incorporated into liposomes, cochleates, biodegradable polymers such as poly-lactide, poly-glycolide and poly-lactide-co-glycolides, or ISCOMS (immunostimulating complexes), and supplementary active ingredients may also be employed.

Antigens of the present invention can also be administered in combination with bacterial toxins and their attenuated derivatives as carrier molecules. Other suitable carrier molecules include serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, immunoglobulin, ovalbumin, polysaccharides (e.g., sepharose, agarose, cellulose), inactive virus particles, and amino acid copolymers. The antigens of the invention can also be administered in combination with lymphokines including, but not limited to, interleukin-2, IFN-γ and GM-CSF. The antigens of the invention can also be expressed in vivo after administration of DNA encoding these antigens.

The vaccines can be administered to a human or animal by a variety of routes, including but not limited to parenteral, intraarterial, intradermal, transdermal (such as by the use of slow release polymers), intramuscular, intraperitoneal, intravenous, subcutaneous, oral and intranasal routes of administration. In a preferred embodiment, the composition is administered orally. The amount of antigen employed in such vaccines will vary depending upon the identity of the antigen. Adjustment and manipulation of established dosage ranges used with traditional carrier antigens for adaptation to the

present vaccine is well within the ability of those skilled in the art. The vaccines of the present invention are intended for use in the treatment of both immature and adult warm-blooded animals, and, in particular, humans. Typically, the antigen and the purified saponin will be administered at the same time.

The oral adjuvant action of purified saponins has a number of important implications. The oral adjuvanticity of purified saponins can increase the concentration of protective antibodies produced against an oral antigen in the vaccinated organism. As a result, effective vaccination can be achieved with a smaller quantity of antigen than would be normally required. This reduction in the required amount of antigen may lead to more widespread use of vaccines which are difficult and costly to prepare. Additionally, the use of purified saponins as oral adjuvants can enhance the ability of antigens which are weakly antigenic or poorly immunogenic, particularly when administered orally, to elicit an immune response. It may also provide for safer vaccination when the antigen is toxic at the concentration normally required for effective oral immunization. By reducing the amount of antigen, the risk of toxic reaction is reduced. It may also provide for safer vaccination by enabling the use of an antigen or vaccine formulation which is safe by oral route but toxic by parenteral route.

Typically, vaccination regimens call for the administration of antigen over a period of weeks or months in order to stimulate a "protective" immune response. A protective immune response is an immune response sufficient to prevent infection or reduce the severity of infection (compared with severity of infection in the absence of the elicited immune response) caused by a particular pathogen or pathogens

to which the vaccine is directed. The use of purified saponins as adjuvants for an oral antigen may reduce the time course of effective vaccination regimens. In some instances, it may result in the generation of a protective response in a single dose. The vaccine compositions of this invention are also useful therapeutically, to reduce the number and severity of symptomatic episodes in subjects already infected with the antigen. The vaccine compositions may also be used as an oral booster immunization for parenterally administered antigens.

Work described herein assessed the ability of a purified saponin, e.g., QS-21, delivered orally or intranasally to serve as an adjuvant to induce an immune response to an antigen, e.g., tetanus toxoid (TT) or ovalbumin (OVA). As described herein, a purified saponin, QS-21, has been used in oral immunizations with TT and OVA, and QS-21 has exhibited adjuvant activity by this delivery route.

The total serum IgG anti-TT antibody responses were comparable to those obtained when cholera toxin (CT) or *E. coli* heat labile toxin (LT) was administered orally to C57BL/6 mice. Interestingly, a dichotomy was observed when immune responses were compared at doses of 50 μg and 100 μg of QS-21 delivered orally with 250 μg of TT. The lower dose of QS-21 induced antigen-specific IgG subclasses of IgG1, IgG2b and IgG3, while the 100 μg QS-21 dose induced IgG2a in addition to the other subclasses. Intranasal administration of QS-21 and TT also led to an adjuvant effect in serum IgG and IgA titers, and results indicate that vaginal wash antigen-specific IgA and IgG titers, while modest, were elevated compared to controls. Since IgG subclass responses are suggestive of T helper cell activity, the results indicate that the 50 μg dose of QS-21 induced a Th2-type

response, while the 100 μg dose induced a Th1-type response. This is further supported by the lower IgE responses induced by 50 μg of QS-21, since IgE is a hallmark of a Th2 response. Thus, it appears that the manipulation of immune responses toward a Th1 or Th2 pathway can be accomplished by alterations in QS-21 dosage.

The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings of all references cited herein are hereby incorporated herein by reference.

EXAMPLES

ORAL ADMINISTRATION OF QS-21 WITH TETANUS TOXOID

As described herein, the immune response to tetanus toxoid (TT), co-orally administered with the saponin QS-21 as an adjuvant has been analyzed. TT was employed as a model antigen because this protein has been used with the mucosal adjuvant cholera toxin (CT) and *E. coli* heat labile toxin (LT), and thus, comparisons could be made as to potency, serum isotype specific responses, serum IgG subclasses, serum IgE antibodies, mucosal IgA antibodies and the cytokine profiles induced by QS-21 with those obtained with CT and LT as standard mucosal adjuvants.

Prior to immunization, C57BL/6 mice were deprived of food for 2 hours, and 30 minutes before oral immunization they received 0.5 ml of a solution consisting of 8 parts Hank's balanced salt solution and 2 parts 7.5% sodium bicarbonate in order to neutralize stomach acidity. Mice received 250 μg of TT at days zero 0, 7, 14 together with 50, 100 or 500 μg of QS-21 in 0.25 ml of PBS by gavage. Serum and fecal extracts

were collected at days 7, 15 and 21 and analyzed for TT-specific antibody responses.

The results showed the orally administered QS-21 induces a systemic immune response to the co-orally given protein antigen (TT). The IgG and IgM responses were detectable from day 7 following the first immunization and were not strongly enhanced by further immunization (Figures 1A and 1B, respectively).

Interestingly, no significant IgA responses were detected in serum. The analysis of immunoglobulin subclasses (Figure 2) revealed that the different doses of QS-21 lead to different patterns of IgG subclasses. Indeed, the lower dose of QS-21 (50 μg) was associated with IgG1, IgG2b and minimal IgG3 antibodies, while 100 and 500 μg doses additionally induced IgG2a antibodies. The differential effect of QS-21 doses was also observed for IgE responses, since responses were detected for both polyclonal (Figure 3A) and TT-specific (Figure 3B) IgE responses with 50 μg QS-21 at day 7, and lower or no IgE responses were detected with 100 and 500 μg of QS-21, respectively.

Analysis of fecal extracts indicated that 3 of 5 mice generated mucosal anti-TT IgA titers of 1:64 at the 50 μg dose of QS-21, and minimal IgA responses were detected at the 100 or 500 μg dosage (Figure 4). IgA is typically considered to be enhanced by Th2 cytokines, again indicating that higher doses of QS-21 down-regulated the Th2 response.

C57BL/6 mice were orally immunized at days 0, 7, and 14 with 250 μg tetanus toxoid and 50 μg QS-21. At day 21, spleen or Peyer's patch were isolated. CD4+ cells were isolated, which were then stimulated with antigen (tetanus toxoid). The antigen stimulated CD4+ cells then secreted cytokines associated with either

CD4+ cells of Th1 type (cytokines IL-2, and interferon-gamma (IFN-g) or of Th2 type (cytokines IL-4, IL-5, IL-6, and IL-10). After QS-21 immunization, both spleen and Peyer's patches secrete both Th1 and Th2 cytokines (Figure 8). This is in contrast to cholera toxin, which is known to induce Th2 type immunity. For example, after oral QS-21/TT, the Th1-associated cytokine IFN-gamma is stimulated to >250 pg/ml in Ag-stimulated CD4 cells from Peyer's patches. By comparison, IFN-gamma is stimulated <25 pg/ml by oral cholera toxin/TT (data not shown).

Figure 9 shows a comparison of cholera toxin (CT) adjuvant and QS-21 adjuvant as oral adjuvants for raising serum antibody titers. A dose of 10 ug cholera toxin was used and a dose of 50 ug QS-21 was used. Serum titers were determined after 3 oral immunizations (days 0, 7, 14), with collection of sera at day 21. Figure 9A shows IgM, IgA and IgG titers, which are generally comparable between the two adjuvants although serum IgA is slightly lower with QS-21 immunization. Figure 9B shows serum IgG isotypes. Again, the serum titers are comparable although IgG1 responses are higher with cholera toxin (consistent with the Th2-induction by this adjuvant).

Another marker of a Th2 response is the serum IgE response. This response, measured at days 7, 14, and 21, is induced by cholera toxin and is largely absent with QS-21 (Figure 10A). Another method of detection of antigen-specific IgE antibody induced in the mice is an assay for passive cutaneous anaphylaxis (PCA). This is measured by sensitization of shaved rat skin by injection of serial dilutions of immune mouse sera, followed by an i.v. injection of antigen in 1% Evan's blue dye in PBS. The rats are killed 10 min after the i.v. injection and the diameter of blueing resulting

from the localized degranulation of mast cells was determined. The endpoint titer was the last dilution of mouse serum resulting in a diameter of blueing of ≥ 5mm. Cholera toxin induced high PCA titers, indicating high tetanus toxoid-specific IgE. In contrast, QS-21 did not induce significant PCA titers (Figure 10B).

Finally, fecal extracts and vaginal washes were examined for tetanus toxoid-specific IgA. Both cholera toxin and QS-21, given with oral tetanus toxoid, induced fecal IgA and vaginal IgA although titers were higher for cholera toxin (Figure 11). These responses are thought to be induced as a Th2 response. The lower IgA titers with QS-21 may be due to the mixed Th1/Th2 cytokine response induced by this adjuvant.

Table 1 shows both the effect of QS-21 of stimulating serum IgG to an oral immunization with tetanus toxoid as well as the positive effect of QS-21 on protection from challenge with tetanus toxin. C57BL/6 mice were immunized orally with tetanus toxoid on days 0, 7, and 14. They received 0, 50 or 100 μg QS-21 as the adjuvant. At day 21, sera for tetanus toxoid-specific IgG was collected. They were then challenged by subcutaneous route with 1 ug or 100 ug of tetanus toxin (1 or 100 minimum lethal doses, respectively).

Table 1. Survival of Mice Orally Immunized with Tetanus Toxoid and QS-21 as Adjuvant to Systemic Challenge with Tetanus Toxin

	0 mg QS-21		50 μg QS-21	100 μg QS-21
Serum IgG (Reciprocal Log_2 Titers)	<10	<10	17	17
Tetanus Toxin (μg/mouse)	1	100	100	100
Number of Survivors per group	0/5	0/3	5/5	3/3

Mice orally immunized on days 0, 7 and 14 with tetanus toxoid (TT) and indicated doses of QS-21 as adjuvant were challenged on day 21 by subcutaneous injection of indicated doses of tetanus toxin in 0.5 ml of PBS-0.2% gelatin. Death of unprotected mice occurred within 48 hours.

Both doses of QS-21 had a significant booster effect on serum IgG (an increase of seven log2 units over that induced in absence of adjuvant). This oral immunization with tetanus toxoid and QS-21 stimulates a systemic immune response that is sufficient to protect against a tetanus toxin challenge. Naive mice injected with 1 or 100 μg of tetanus toxin by subcutaneous route typically die within 48 hours. Mice that were orally immunized with tetanus toxoid in absence of QS-21 were not protected from challenge (0 survivors/5 mice receiving 1 μg tetanus toxin challenge and 0/3 mice receiving 100 ug tetanus toxin challenge). However, the addition of either 50 or 100 μg QS-21 to the tetanus toxoid vaccine was then sufficient to protect all mice against challenge with the more rigorous challenge (100 minimum lethal doses of tetanus toxin).

Figure 12 shows that QS-21 can act as an oral adjuvant for enhancement of systemic and mucosal immune response to other orally administered antigens. C57BL/6 mice were orally immunized at days 0, 7, and 14 with 500

μg ovalbumin with or without 50 μg QS-21. QS-21 administration resulted in enhanced serum IgM, IgG, IgA, IgG1 and IgG2b titers and fecal IgA titers on samples that were collected at day 21.

INTRANASAL DELIVERY OF QS-21

As described herein, two different protocols to demonstrate the adjuvant properties of QS-21 given intranasally with TT were employed. The first protocol consisted of administering 20 μg TT alone, or 20 μg TT given with 20 or 40 μg of QS-21 to 3 groups of 5 mice. Doses of 15 μl were given in PBS on day 0, 14, and 28. The total serum IgG and subclass endpoint titers at day 42 are shown in Figure 5.

While TT alone gave an IgG endpoint titer of approximately 1:100,000, the titers of mice given either dose of QS-21 were 1:2 million. The adjuvant effect was distributed throughout all IgG subclasses. While IgG1 and IgG2b subclasses predominated, substantial levels of IgG2a and IgG3 were also detected. The serum TT-specific IgA endpoint titers are illustrated in Figure 6.

Again, an adjuvant effect was observed with either dose of QS-21, and the endpoint titer of 1:4,000 at day 35 was elevated when compared to a titer of 1:512 for TT given alone. The presence of TT-specific IgA (Figure 7A) and IgG (Figure 7B) in vaginal wash samples was determined employing 150 μl PBS as the wash fluid. Low, but significant, antigen-specific antibodies of both isotypes were observed, and enhanced titers were associated with either dose of QS-21, when compared with TT given alone.

A second intranasal protocol was initiated during the course of the initial protocol. In this protocol, groups of 5 mice were intranasally immunized with 50 μg

TT with and without 40 μg of QS-21 on days 0, 5 and 14. It is clear that a) the total antigen-specific IgG levels will be similar to Figure 5; b) the IgG subclass responses were predominately composed of IgG1 and IgG2b, although IgG2a is also detected; and c) vaginal IgA is elevated in mice given QS-21 (1:32) versus control (1:8). The levels of anti-TT fecal IgA were also examined in this experiment. Intranasal immunization resulted in an endpoint titer of 1:128 on day 21 with TT only and was not further elevated in QS-21-treated mice.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed in the scope of the invention.

CLAIMS

We claim:

1. A composition suitable for oral administration comprising an adjuvant amount of a purified saponin, and optionally comprising a physiologically acceptable vehicle.

2. A composition according to Claim 1, further comprising an antigen.

3. A composition according to Claim 1, wherein the purified saponin is selected from the group consisting of QS-7, QS-17, QS-18, QS-21 and combinations thereof.

4. A composition according to Claim 2, wherein the antigen is a bacterial antigen.

5. A composition according to Claim 1, wherein the composition elicits a systemic immune response comprising both Th1- and Th2-type responses in a vertebrate to whom it is administered.

6. A composition according to Claim 1, wherein the composition elicits a mucosal immune response in a vertebrate to whom it is administered.

7. An immunogenic composition suitable for oral administration comprising an adjuvant amount of a purified saponin, and optionally comprising a physiologically acceptable vehicle.

8. An immunogenic composition according to Claim 7, further comprising an antigen.

9. An immunogenic composition according to Claim 7, wherein the purified saponin is selected from the group consisting of QS-7, QS-17, QS-18, QS-21 and combinations thereof.

10. An immunogenic composition according to Claim 8, wherein the antigen is a bacterial antigen.

11. An immunogenic composition according to Claim 7, wherein the composition elicits a systemic immune response comprising both Th1- and Th2-type responses in a vertebrate to whom it is administered.

12. An immunogenic composition according to Claim 7, wherein the composition elicits a mucosal immune response in a vertebrate to whom it is administered.

13. A vaccine composition suitable for oral administration comprising an adjuvant amount of a purified saponin and an antigen, and optionally comprising a physiologically acceptable vehicle.

14. A method of eliciting an immune response in a vertebrate host to an orally administered antigen, comprising orally administering to a vertebrate host an antigen and an adjuvant amount of a purified saponin, and wherein the antigen and/or the purified saponin is optionally administered with a physiologically acceptable vehicle.

15. A method according to Claim 14, wherein the immune response is protective against challenge with a pathogen from which the antigen is derived.

16. A method of enhancing a non-specific immune response in a vertebrate host, comprising orally administering to a vertebrate host an effective amount of a purified saponin along with an optional physiologically acceptable vehicle.

17. A method according to Claim 16, wherein the immune response is a mucosal immune response.

18. A method according to Claim 16, wherein the immune response is a systemic immune response comprising both Th1- and Th2-type responses.

19. An immunogenic composition suitable for oral administration comprising an adjuvant amount of QS-21 and tetanus toxoid, and optionally comprising a physiologically acceptable vehicle.

20. A vaccine composition suitable for oral administration comprising an adjuvant amount of QS-21 and tetanus toxoid, and optionally comprising a physiologically acceptable vehicle.

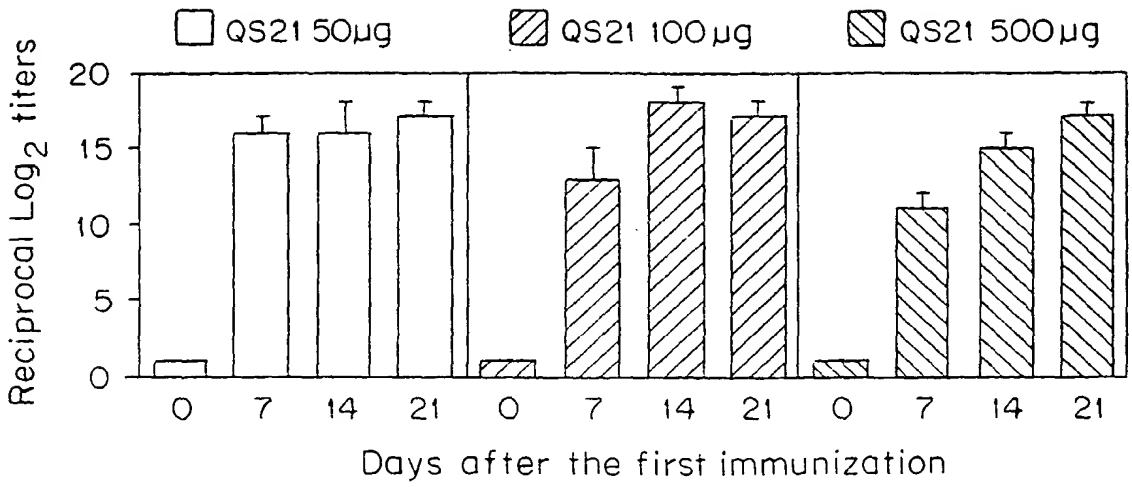

FIG. 1A

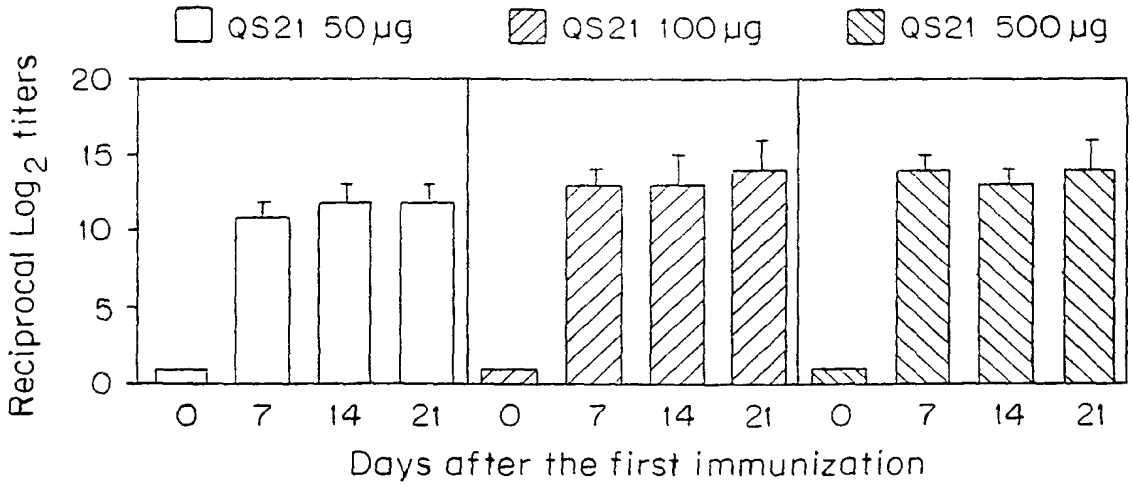

FIG. 1B

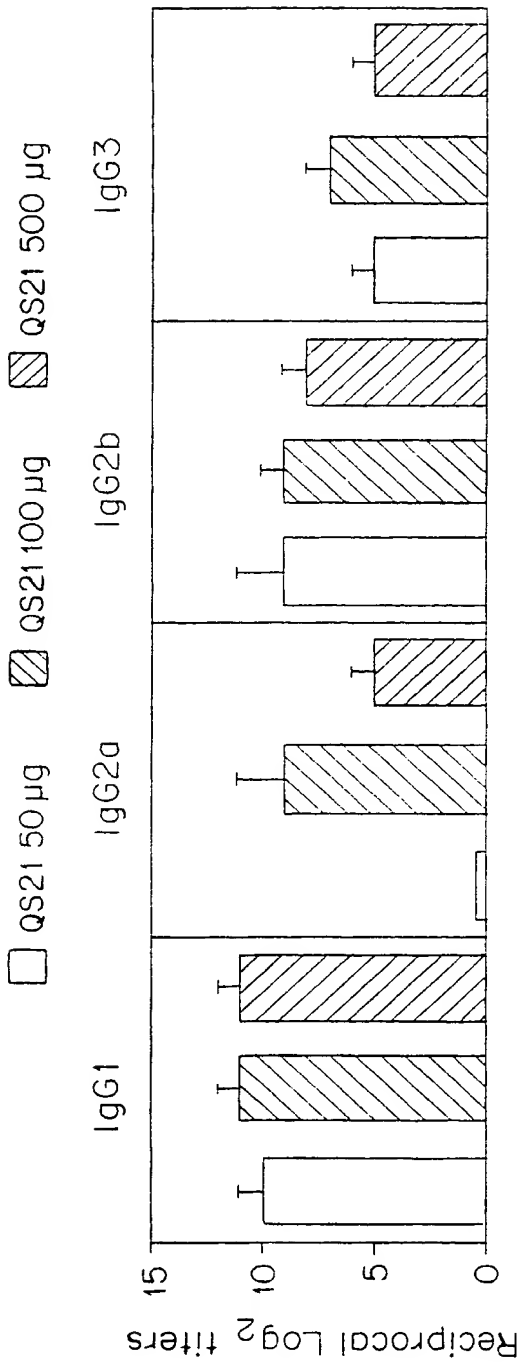

FIG. 2

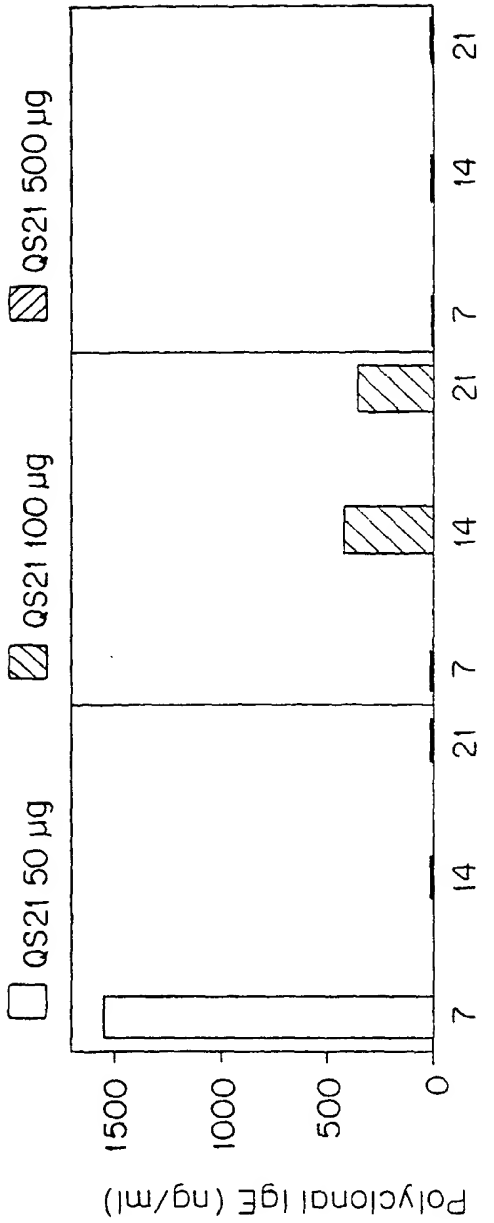

FIG. 3A

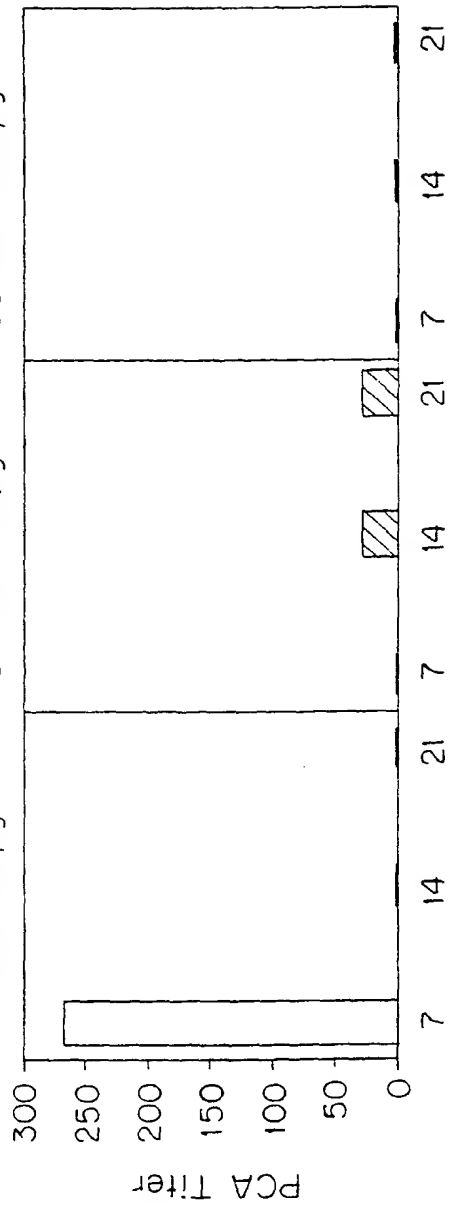

FIG. 3B

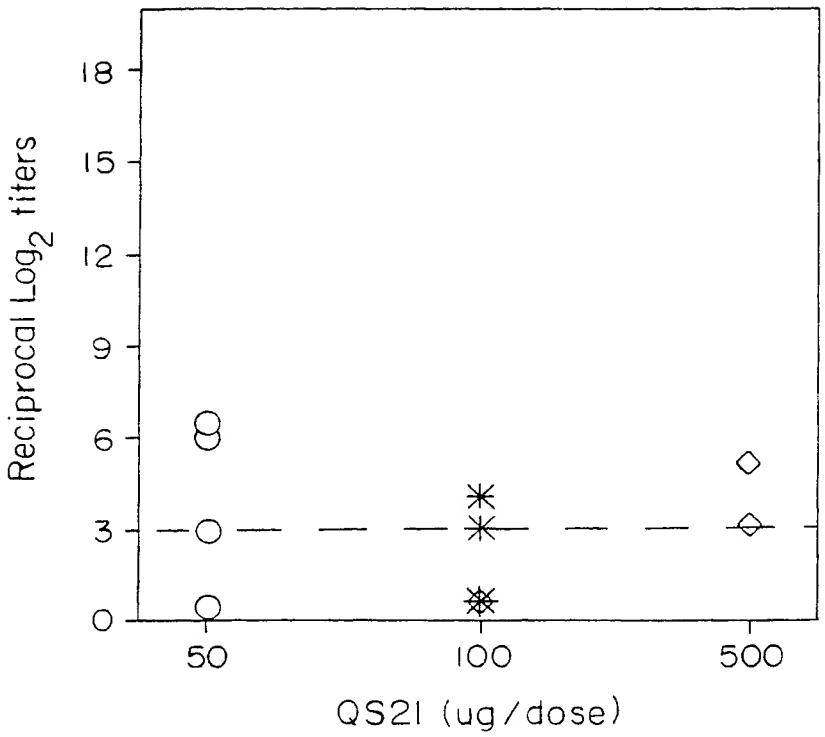

FIG. 4

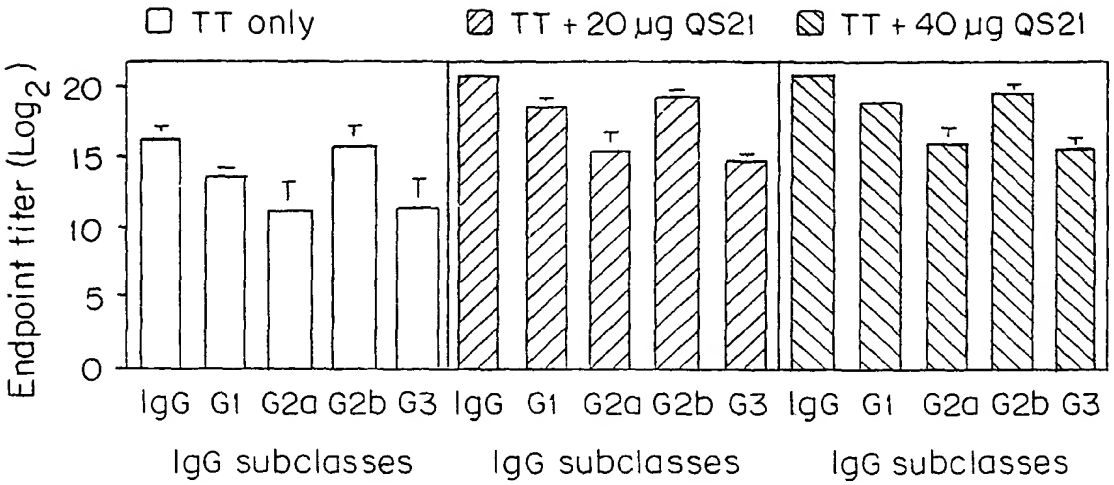

FIG. 5

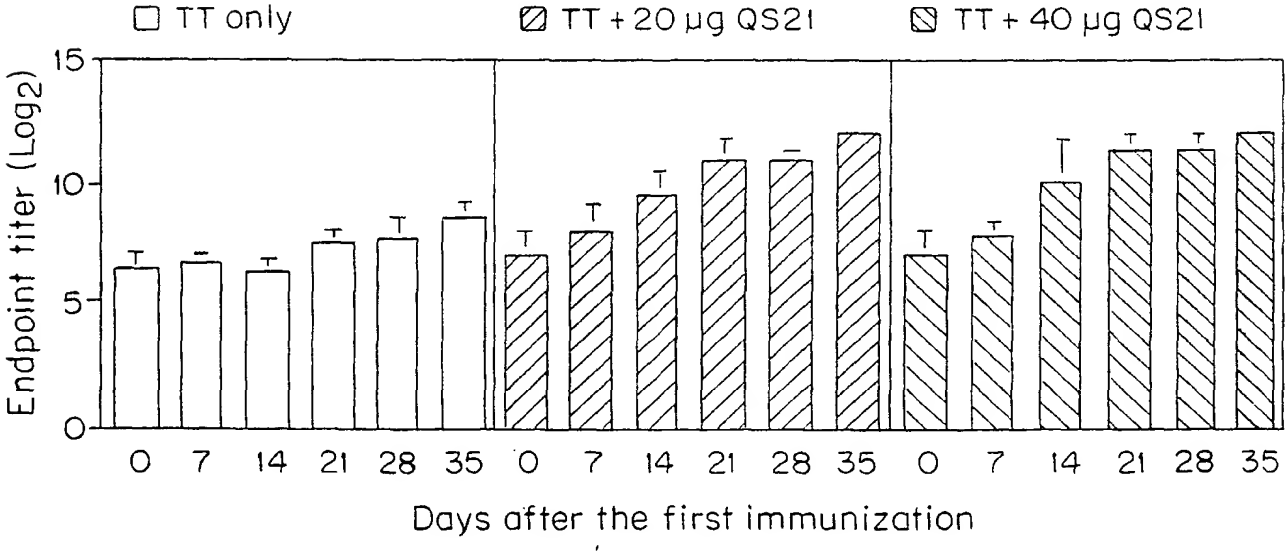

FIG. 6

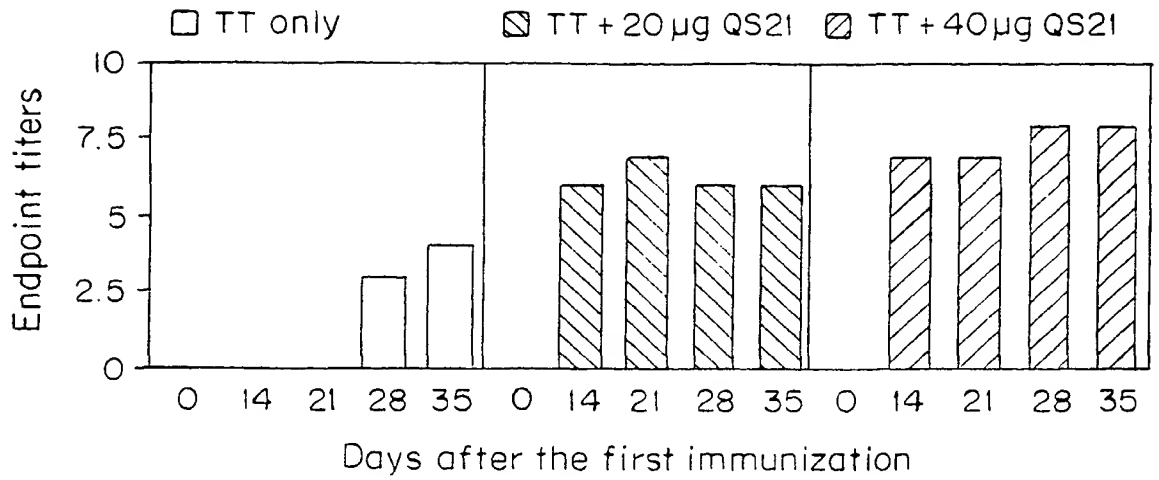

FIG. 7A

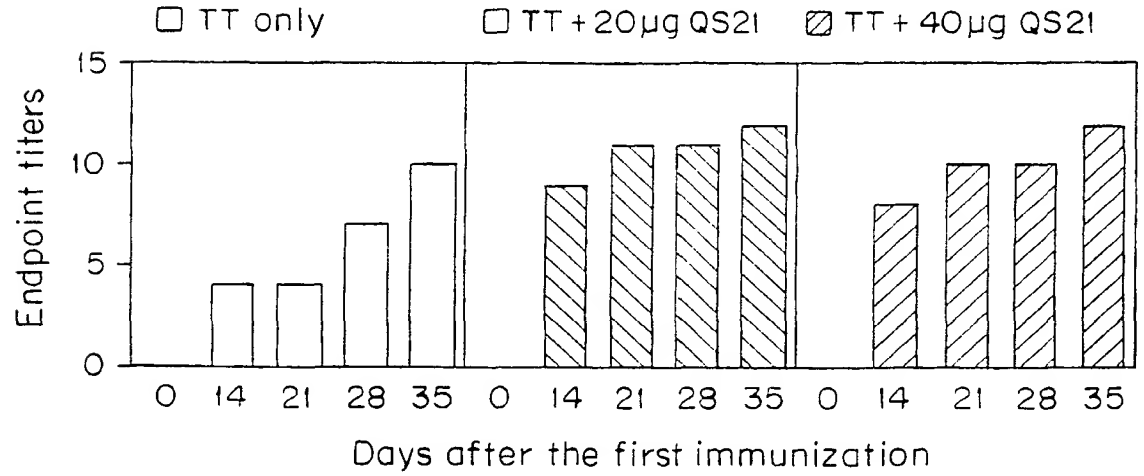

FIG. 7B

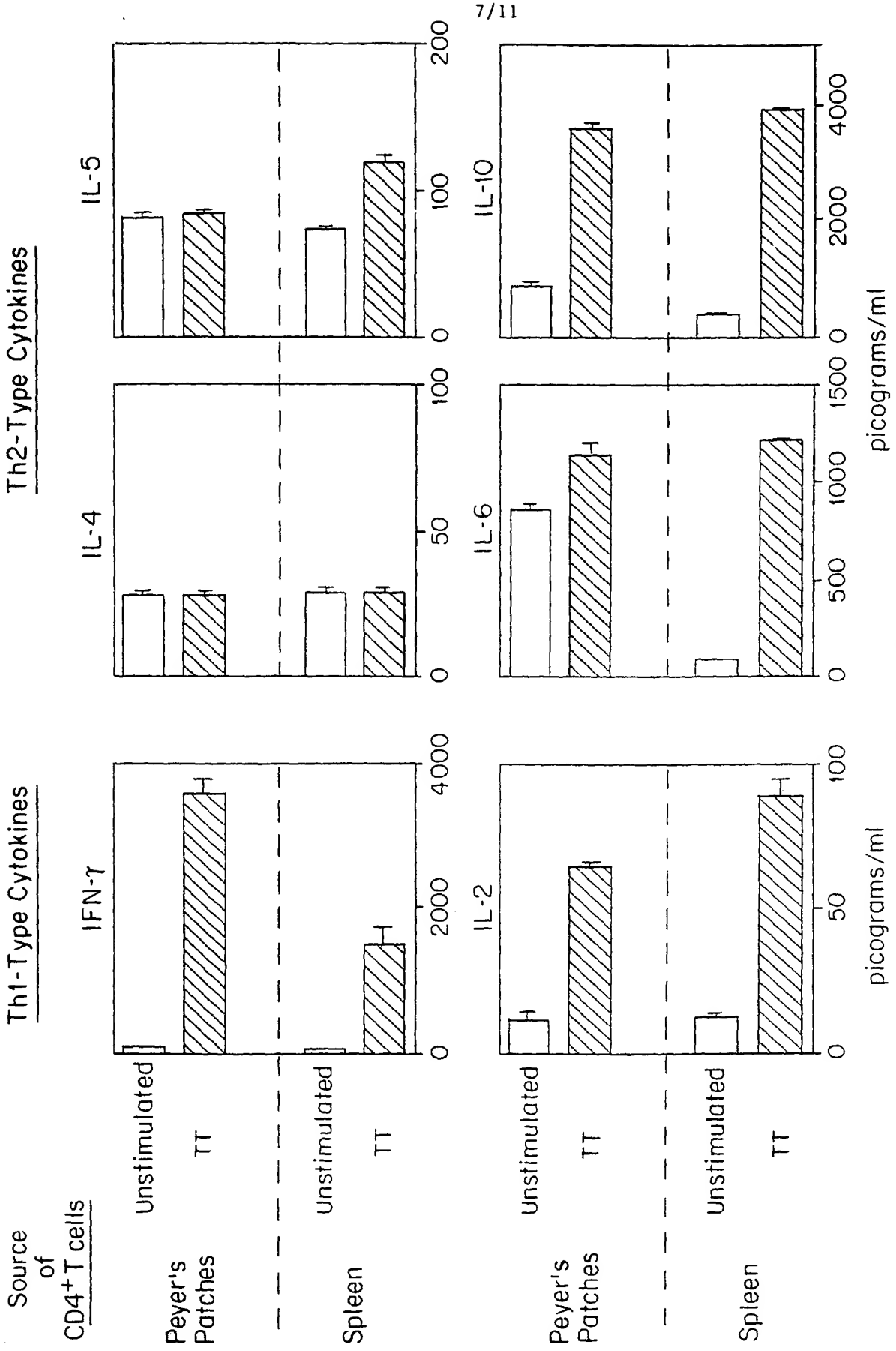

FIG. 8

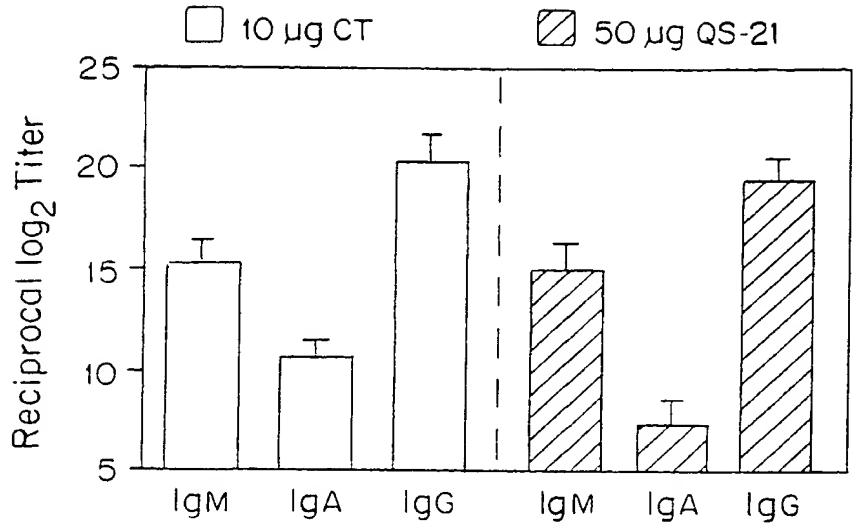

FIG. 9A

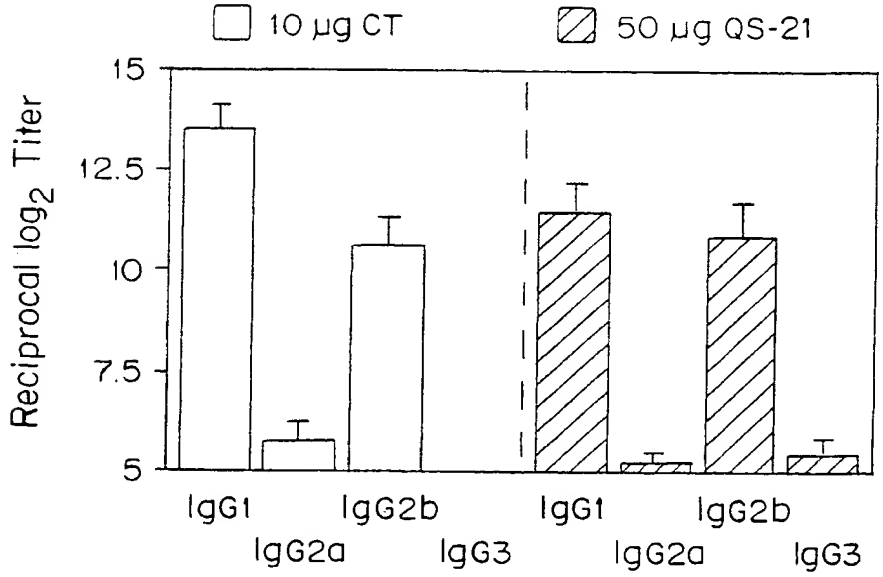

FIG. 9B

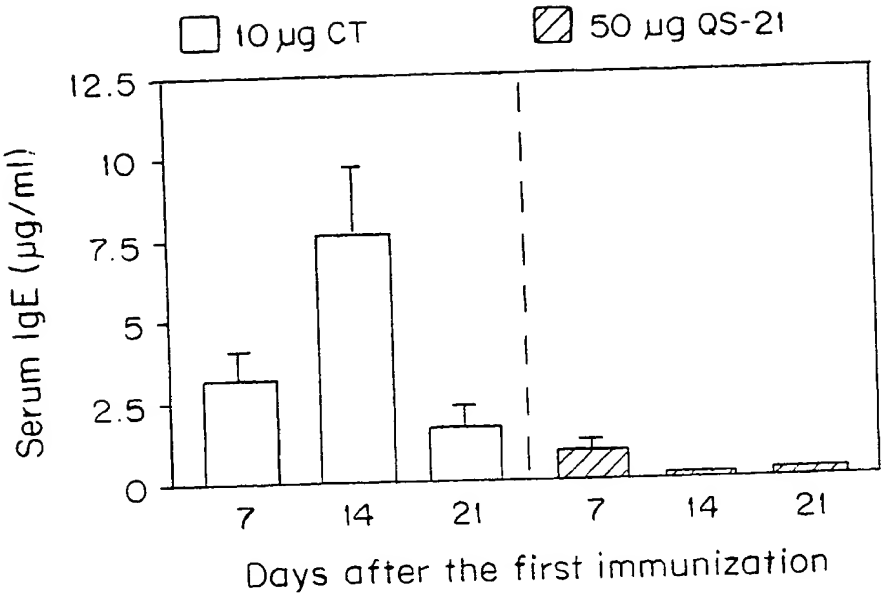

FIG. 10A

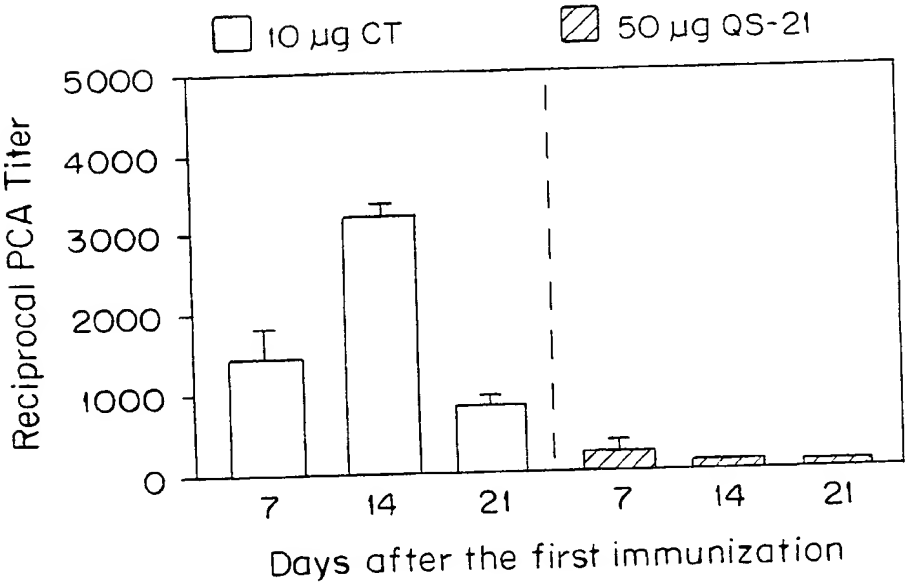

FIG. 10B

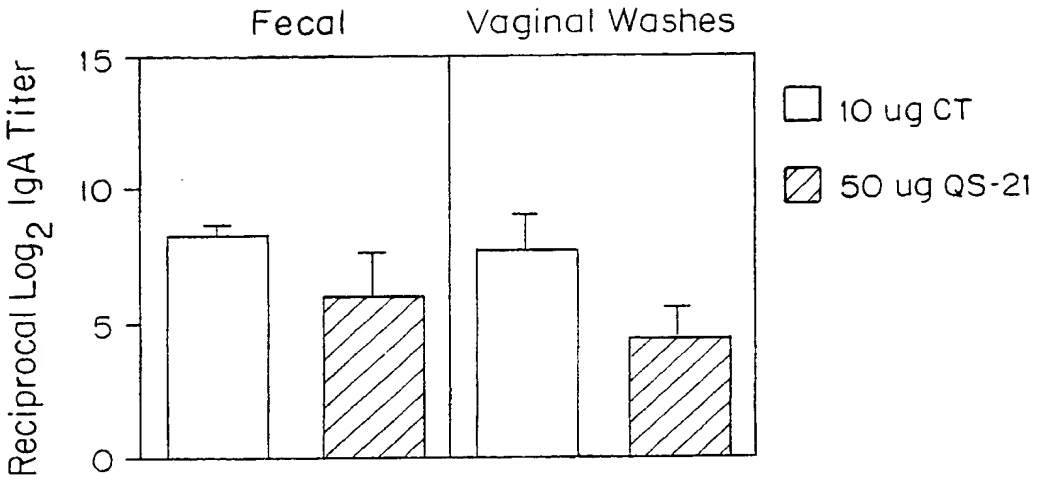

FIG. 11

ORAL QS-21: MUCOSAL AND SYSTEMIC IMMUNE RESPONSES TO OVA

Oral vaccine: Administered at days 0, 7, 14
Ovalbumin (OVA): 500 μg
QS-21:50 μg

	OVA (Day 21) (Reciprocal Log2 Titers)	OVA/QS-21 (Day 21) Reciprocal Log2 Titers)
IgM	12	14
IgG	14	18
IgA	5	8
IgE (polyHRP)	<5	<5
IgG1	8	16
IgG2a	<5	<5
IgG2b	6	9
IgG3	<5	<5
Fecal IgA	<2	6

FIG. 12

INTERNATIONAL SEARCH REPORT

National Application No
PCT/US 98/11603

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/39 A61K39/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOYAKA P.N. ET AL.: "Orally deliverd saponin-derived QS-21 adjuvant elicits systemic and mucosal antibody responses to co-admininistered protein antigen" THE JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, vol. 99, no. 1, part 2, January 1997, page S35 XP002075674 A145 see the whole document ---	1-20
X	US 5 597 807 A (ESTRADA ALBERTO ET AL) 28 January 1997 cited in the application see column 1, line 8-11 see column 2, line 31-51 see column 3, line 55-66 see table 1 --- -/--	1,2,4, 6-8,10, 12-17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
28 August 1998	10/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication.where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 05789 A (CAMBRIDGE BIOTECH CORP) 1 April 1993 see page 13, line 26 - page 14, line 20 see page 17, line 22 - page 18, line 2 see page 18, line 21 - page 19, line 5	1-4, 6-10,12, 13,19,20
X	WO 88 09336 A (CAMBRIDGE BIOSCIENCE CORP) 1 December 1988 see page 1, line 16-19 see page 6, line 13-22 see page 16, line 24 - page 17, line 7 see claims 19-28	1-3,6-9, 12,13
A	MOWAT A. ET AL.: "Immune-stimulating complexes as adjuvants for inducing local and systemic immunity after oral immunization with protein antigens" IMMUNOLOGY, vol. 80, 1993, pages 527-534, XP002075677 see abstract see page 533, line 29-55	1-20
A	SJÖLANDER A. ET AL: "Iscoms containing purified Quillaja saponins upregulate both Th1-like and Th2-like immune responses" CELLULAR IMMUNOLOGY, vol. 177, 1997, pages 69-76, XP002075675 see abstract see page 69, right-hand column, line 13-25 see page 75, right-hand column, line 12-46	1-20
A	SOLTYSIK S. ET AL.: "Structure/function studies of QS-21 adjuvant: assessment of triterpene aldehyde and glucuronic acid roles in adjuvant function" VACCINE, vol. 13, no. 15, 1995, pages 1403-1410, XP002075676 see abstract	1-20

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 11603

Box I Observations wh r certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 14-18
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 14-18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

National Application No
PCT/US 98/11603

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5597807 A	28-01-1997	AU 3338495 A	04-03-1996
		CA 2196082 A	15-02-1996
		WO 9603998 A	15-02-1996
		EP 0773786 A	21-05-1997
		JP 10503495 T	31-03-1998
		US 5688772 A	18-11-1997
WO 9305789 A	01-04-1993	US 5583112 A	10-12-1996
		AU 662562 B	07-09-1995
		AU 2666492 A	27-04-1993
		CA 2118928 A	01-04-1993
		EP 0606317 A	20-07-1994
		JP 7504156 T	11-05-1995
		NO 940949 A	16-05-1994
		NZ 244410 A	28-03-1995
WO 8809336 A	01-12-1988	AT 116993 T	15-01-1995
		AU 616670 B	07-11-1991
		AU 1934088 A	21-12-1988
		CA 1331443 A	16-08-1994
		DE 3852761 D	23-02-1995
		DE 3852761 T	07-09-1995
		DK 602989 A	29-11-1989
		EP 0362279 A	11-04-1990
		JP 2504266 T	06-12-1990
		JP 2731563 B	25-03-1998
		US 5057540 A	15-10-1991
		US 5583112 A	10-12-1996